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(54) Title: METHODS FOR DIAGNOSIS AND TREATMENT OF HUMAN DISEASES INCLUDING HYPERTENSION

(57) Abstract: Methods for diagnosis and treatment of human disease, particularly human disease characterised by abnormal cytosolic ion composition in diseased cells resulting from reduced or over activity of Na^+ transport proteins (e.g. hypertension), are disclosed. Additionally, the specification discloses novel Na^+ receptors and isolated DNA molecules encoding same.

METHODS FOR DIAGNOSIS AND TREATMENT OF HUMAN DISEASES INCLUDING HYPERTENSION

Field of the Invention:

The present invention relates to the diagnosis and treatment of human disease, particularly human disease characterised by abnormal cytosolic ion composition resulting from reduced or over activity of Na^+ transport proteins such as the ubiquitous Na^+-H^+ exchanger, NHE1.

Background of the Invention:

In recent years, over activity of Na^+ transporting systems in absorptive epithelia has been implicated in the pathogenesis of a number of major diseases including hypertension (1, 2), diabetic nephropathy (3), cardiological syndrome X (4), ventricular hypertrophy (5), chronic pulmonary hypertension (6) and cystic fibrosis (7). In the case of the hereditary hypertensive disease known as Liddle's syndrome, this activity has been attributed to a mutation of the epithelial Na^+ channel leading to loss of an inhibitory feedback mechanism which normally switches off Na^+ channel activity in response to increased intracellular Na^+ (8, 9). The mechanisms that underlie this so-called homocellular regulation have been the subject of controversy, but recent experiments have revealed a previously unsuspected mechanism in which cytosolic Na^+ is "sensed" by an intracellular receptor (10). This receptor activates the G protein, G_o (11), the α-subunit of which then causes the ubiquitin-protein ligase, Nedd4 (10), to ubiquitinate and inactivate the epithelial Na^+ channels (12, 13). This receptor for intracellular Na^+ is blocked by amiloride and amiloride analogs such as dimethylamiloride and benzimidazole guanidinium (10), thus explaining the previously puzzling ability of these agents to stimulate Na^+ channel activity (14).

The present applicants have now found that the intracellular Na^+ receptor that controls absorptive epithelial Na^+ channels also controls the activity of the ubiquitous isoform of the Na^+-H^+ exchanger 1, NHE1 (34). This finding suggests that intracellular Na^+ receptors form part of a general mechanism for regulating Na^+ transport proteins. It is therefore anticipated that the intracellular Na^+ receptors (and the signal-transduction systems by which they control Na^+ channels, Na^+-H^+ exchangers and other Na^+

transporting proteins) shall provide a useful target for diagnostic assays and treatments for hypertension and other diseases.

Disclosure of the Invention:

Thus, in a first aspect, the present invention provides a method of treatment of a human disease which is characterised by abnormal cytosolic ion composition in diseased cells resulting from reduced or over activity of a Na^+ transport protein, the method comprising administering to a subject having said disease an effective amount of an agent that substantially restores the ion composition of the cytosol in said diseased cells to that which is found in corresponding cells from healthy tissue.

Preferably, the present invention provides a method of treatment of a human disease which is characterised by abnormal cytosolic ion composition in diseased cells resulting from reduced or over activity of a Na^+ transport protein other than an epithelial Na^+ receptor. Most preferably, the present invention provides a method of treatment of a human disease which is characterised by abnormal cytosolic ion composition in diseased cells resulting from reduced or over activity of a Na^+ transport protein selected from those which are inactivated by ubiquitination (e.g. through the action of a ubiquitin-protein ligase) and, particularly, from those included in the group consisting of NHE1, Na^+-H^+ exchanger 2 (NHE2) (35), Na^+-H^+ exchanger 3 (NHE3) (36), the Na^+-HCO_3^- cotransporter (37) and the Na^+K^+2Cl cotransporter (38).

Where the characteristic abnormal cytosolic ion composition arises from reduced Na^+ transport protein activity resulting from, for example, Na^+ transport protein mutation (e.g. hereditary), depressed Na^+ transport protein expression or inappropriate activity of the Na^+ transport protein inhibitory feedback mechanism, the administered agent may be selected from gene therapy agents (e.g. recombinant adenoviruses capable of causing the expression of non-mutated Na^+ transport protein) and agents capable of blocking the Na^+ transport protein inhibitory feedback mechanism. Preferred agents of the latter kind are amiloride and amiloride analogs (e.g. 6-iodoamiloride, N-dimethylamiloride, and benzimidazoylguanidium), G-protein inhibitors (e.g. GDP-β-S (39) and NF023 (40)) and agents that inhibit the action of ubiquitin protein ligase on the Na^+ transport protein. Examples of this latter kind of agents are dominant negative mutants of ubiquitin (e.g.

K48R (24)), agents that prevent binding of the ubiquitin protein ligase to the Na^+ transport protein (e.g. membrane permeable peptide analogs of the protein motif to which the ubiquitin protein ligase binds such as the WW2 and WW3 domains of Nedd4 (10)), agents that prevent ubiquitination of the Na^+ transport protein (e.g. membrane permeable peptide analogs of the protein motif which is actually ubiquitinated, such as the N-terminal of the α- or γ-subunit of ENaC (41)) and inhibitors of the effectors of ubiquitin action on the Na^+ transport protein including proteins involved in endocytosis (e.g. membrane permeable analogs of amphiphysin SH3 peptide(42)), and inhibitors of the degradation of the Na^+ transport protein by proteasomes (e.g. lactacystin) or lysosomes (e.g. bafilomycin or chloroquine). Peptide analogs may be made to be membrane permeant by including a *Drosophila* antennapedia homeobox domain (15, 16).

Where the characteristic abnormal cytosolic ion composition arises from Na^+ transport protein over activity resulting from, for example, Na^+ transport protein mutation (e.g. hereditary), loss of the Na^+ transport protein inhibitory feedback mechanism or inappropriate activity of other control systems (e.g. excessive levels of growth factors or glucose), the administered agent may be selected from gene therapy agents (e.g. adenoviruses capable of causing the expression of a protein participating in the Na^+ transport protein inhibitory feedback mechanism), intracellular Na^+ receptor activators (e.g. guanidium and guanidium analogs), G-protein activators (e.g. GTP-γ-S (43) and receptor mimetic peptides such as APP20(17)), ubiquitin ligase activators (e.g. membrane permeable peptides that mimic the effect of active G proteins on the ubiquitin protein ligase), and agents that trigger endocytosis.

An "effective amount" of the agent used in the method of the first aspect will depend upon the particular agent used, however, generally, the amount would be expected to be below about 10 mg/kg. For example, an effective amount of amiloride or an amiloride analog would typically be in the range of 1 to 3 mg/kg.

The agent may be formulated with various pharmaceutically-acceptable excipients and/or carriers commonly used in the art and prepared for administration orally (e.g. as tablets, capsules, caplets or liquids), nasally (e.g. aerosol sprays), rectally (e.g. as suppositries) and transdermally (e.g. as a transdermal patch or dermally absorbed cream or lotion). The agent may also

be formulated as an injectible solution or suspension for subcutaneous, intravenous or intramuscular administration.

In a second aspect, the present invention provides a method of diagnosis of a human disease which is characterised by abnormal cytosolic ion composition in diseased cells resulting from reduced or over activity of a Na^+ transport protein, the method comprising isolating from a subject suspected of having said disease a sample of cells (such as epithelial cells or lymphocytes) and assessing said sample of cells for reduced or over activity of said Na^+ transport protein or its inhibitory feedback mechanism.

Preferably, the present invention provides a method of diagnosis of a human disease which is characterised by abnormal cytosolic ion composition resulting from reduced or over activity of a Na^+ transport protein other than an epithelial Na^+ receptor. Most preferably, the present invention provides a method of diagnosis of a human disease which is characterised by abnormal cytosolic ion composition resulting from reduced or over activity of a Na^+ transport protein selected from those which are inactivated by ubiquitination (e.g. through the action of a ubiquitin-protein ligase) and, particularly, from those included in the group consisting of NHE1, NHE2, NHE3, the Na^+-HCO_3^- cotransporter and the Na^+K^+2Cl cotransporters.

The sample of cells may be assessed for reduced or over activity of Na^+ transport protein by, for example, determining the rate of Na^+-dependent intracellular pH (pH_i) recovery and comparing the value against similarly measured values from cells from healthy tissue isolated from the said suffering subject or from a control (i.e. non-diseased) subject or subjects (e.g. an average value from a panel of two or more healthy subjects).

In a variation of the invention according to the second aspect, the sample of diseased cells may be assessed for over or under expression of the Na^+ transport protein or another protein participating in the Na^+ transport protein inhibitory feedback mechanism (e.g. by polymerase chain (PCR) techniques, Northern blot hybridisation, Western blot or immunoprecipitation).

In a third aspect, the present invention provides a method of diagnosis of a human disease which is characterised by abnormal cytosolic ion composition in diseased cells resulting from reduced or over activity of a Na^+ transport protein, the method comprising isolating a genomic DNA sample from a subject suspected of having said disease and assessing said sample for

the presence of a gene encoding a mutated product causitive of said reduced or over activity of said Na^+ transport protein.

In a fourth aspect, the present invention provides a method of assessing a subject for a predisposition to a human disease which is characterised by abnormal cytosolic ion composition in diseased cells resulting from reduced or over activity of a Na^+ transport protein, the method comprising isolating a genomic DNA sample from a subject and assessing said sample for the presence of a gene encoding a mutated product causitive of reduced or over activity of said Na^+ transport protein.

In the methods of the third and fourth aspects, the human disease is preferably one which is characterised by abnormal cytosolic ion composition resulting from reduced or over activity of a Na^+ transport protein other than an epithelial Na^+ receptor. Preferably, the Na^+ transport protein is selected from the group consisting of NHE1, NHE2, NHE3, the Na^+-HCO_3^- cotransporter and the Na^+K^+2Cl cotransport protein). The genomic DNA sample may be isolated using routine protocols known to the art. The genomic DNA sample may be isolated from any cell sample such as whole blood, tissue biopsy or cheek cell sample. The assessment of the presence of a gene encoding a mutated product causitive of reduced or over activity of the Na^+ transport protein, may be preferably achieved by hybridisation or PCR techniques using probes/primers designed to specifically hybridise to genes including mutated nucleotide sequences. The gene whose presence is to be assessed may encode a mutated Na^+ transport protein or a mutated protein participating in the Na^+ transport protein inhibitory feedback mechanism (e.g. a mutated G-protein or mutated intracellular Na^+ receptor).

The methods of the invention are applicable to, for example, hypertension, renal failure, cardiac hypertrophy and cardiological syndrome X.

The present applicants have also found that the intracellular Na^+ receptor controlling NHE1 is blocked by amiloride and amiloride analogs with the following order of potency:
6-iodoamiloride (EC_{50} = 0.1 μmol/l) < amiloride (1.0 μmol/l) < 5-N-dimethylamiloride (30 μmol/l), benzamil (> 30 μmol/l) < benzimidazolylguanidium (300 μmol/l)

Knowledge of these differing potencies enables the isolation of a DNA molecule encoding the intracellular Na^+ receptor controlling NHE1. That is,

by using the α-subunit of G_o as "bait" in a yeast two-hybrid technique ("The yeast two-hybrid system" edited by P.L. Bartel & S. Fields, Oxford University Press, Oxford, 1997), DNA molecules encoding interacting proteins may be isolated from suitable cDNA or genomic DNA libraries and then screened for the ability of the encoded proteins to bind 6-iodoamiloride. Further screens may be conducted for the relative inability of the encoded proteins to bind benzamil, the ability of antibodies raised to the encoded proteins to immunoprecipitate the α-subunit of G_o, and the ability of antibodies raised to the encoded proteins to block the NHE1 inhibitory feedback mechanism.

By using the yeast two-hybrid system with a constitutively active mutant of the α-subunit of G_o, it is possible to identify and isolate proteins which interact with active G_o and hence are involved in the inhibitory feedback mechanism at a loci downstream of G_o. Similarly, by using the yeast two-hybrid system with a dominant negative mutant of the α-subunit of G_o, it is possible to identify and isolate proteins such as the intracellular Na^+ receptors which are involved in the inhibitory feedback mechanism at a loci upstream of G_o.

The present applicants have isolated 5 cDNA molecules from mouse kidney and mandibular gland cDNA libraries encoding likely intracellular Na^+ receptors controlling NHE1 and Na^+ channels. The 5 candidates are nucleobindin (18), GAIP (19), rap1GAP (20) and novel proteins designated GILT (formerly designated GILT) and SCunique.

Thus, in a fifth aspect, the present invention provides an isolated DNA molecule encoding an intracellular Na^+ receptor designated GILT, said DNA molecule comprising a nucleotide sequence substantially corresponding to that shown as SEQ ID NO: 1 or a nucleotide sequence showing ≥ 75% (more preferably ≥ 85%, most preferably ≥ 95%) homology to that shown as SEQ ID NO: 1.

Preferably, the isolated DNA molecule of the fifth aspect encodes a protein comprising an amino acid sequence substantially corresponding to that shown as SEQ ID NO: 2.

In a sixth aspect, the present invention provides an isolated DNA molecule encoding an intracellular Na^+ receptor designated SCunique, said DNA molecule comprising a nucleotide sequence substantially corresponding to that shown as SEQ ID NO: 3 or a nucleotide sequence showing ≥ 75%

(more preferably ≥ 85%, most preferably ≥ 95%) homology to that shown as SEQ ID NO: 3.

Preferably, the isolated DNA molecule of the sixth aspect encodes a protein comprising an amino acid sequence substantially corresponding to that shown as SEQ ID NO: 4.

The isolated DNA molecule of the fifth and sixth aspect may be incorporated into plasmids or expression vectors (including viral vectors), which may then be introduced into suitable bacterial, yeast, insect and mammalian host cells. Such host cells may be used to express the receptor encoded by the isolated DNA molecule.

Accordingly, in a seventh aspect, the present invention provides a mammalian, insect, yeast or bacterial host cell transformed with the DNA molecule of the fifth or sixth aspect.

In an eighth aspect, the present invention provides a method of producing an intracellular Na^+ receptor, comprising culturing the host cell of the seventh aspect under conditions enabling the expression of the DNA molecule and optionally recovering the expressed receptors.

Preferably, the host cell is mammalian, amphibian or of insect origin. Where the cell is mammalian, it is presently preferred that it be a Chinese hamster ovary (CHO) cell or human embryonic kidney 293 cell. Where the cell is of amphibian origin, it is presently preferred that it be a *Xenopus* oocyte. Finally, where the cell is of insect origin, it is presently preferred that it be an insect Sf9 cell.

In a ninth aspect, the present invention provides an intracellular Na^+ receptor designated GILT, said receptor comprising an amino acid sequence substantially corresponding to that shown as SEQ ID NO: 2, in a substantially pure form.

In a tenth aspect, the present invention provides a candidate intracellular Na^+ receptor designated SCunique, said receptor comprising an amino acid sequence substantially corresponding to that shown as SEQ ID NO: 4, in a substantially pure form.

In an eleventh aspect, the present invention provides an antibody which specifically binds to a receptor according to the ninth or tenth aspect. Such antibodies may be polyclonal or monoclonal and may be produced in accordance with any of the known techniques in the art.

The present applicants have also identified two variants of the nucleotide sequence encoding GILT (SEQ ID NO: 5 and SEQ ID NO: 6) and isolated and sequences some of the 5' non-coding sequence of the nucleotide sequence encoding SCunique (SEQ ID NO: 7). It is to be understood that the present invention extends to these additional nucleotide sequences.

In a twelfth aspect, the present invention provides a method for detecting agonist or antagonist agents of the receptor of the ninth or tenth aspect, comprising contacting said receptor, or a host cell transformed with and expressing the DNA molecule of the fifth or sixth aspect, with a test agent under conditions enabling the activation of said receptor, and detecting an increase or decrease in activity of the receptor.

In a further aspect, the present invention provides a nucleic acid probe/primer comprising a nucleotide sequence of 10 or more nucleotides capable of specifically hybridising to a unique sequence within a DNA molecule having a nucleotide sequence as shown as SEQ ID NO: 1 or SEQ ID NO: 3 under high stringency conditions.

As used herein, the term "high stringency conditions" refers to conditions that (i) employ low ionic strength and high temperature for washing, for example, 15 mM NaCl/1.5 mM sodium citrate/0.1% $NaDodSO_4$ at 50°C; (ii) employ during hybridisation a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C; or (iii) employ 50% formamide, 5 x SSC (750 mM NaCl, 75 mM sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS and 10% dextran sulfate at 42°C in 0.2 x SSC (30 mM NaCl, 3 mM sodium citrate) and 0.1% SDS.

The term "substantially corresponding" as used herein in relation to nucleotide sequences is intended to encompass minor variations in the nucleotide sequence which due to degeneracy in the DNA code do not result in a change in the encoded protein. Further, this term is intended to encompass other minor variations in the sequence which may be required to enhance expression in a particular system but in which the variations do not result in a decrease in biological activity of the encoded protein.

The term "substantially corresponding" as used herein in relation to amino acid sequences is intended to encompass minor variations in the

amino acid sequences which do not result in a decrease in biological activity of the encoded protein. These variations may include conservative amino acid substitutions. The substitutions envisaged are:-

G, A, V, I, L, M; D, E; N, Q; S, T; K, R, H; F, Y, W, H; and P, Nα-alkylamino acids.

References to percent homology values herein are calculated by the BLAST program blastn as described by Altschul, S.F. et al., "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Research Vol. 25, No. 17, pp 2289-3402 (1997).

The terms "comprise", "comprises" and "comprising" as used throughout the specification are intended to refer to the inclusion of a stated step, component or feature or group of steps, components or features with or without the inclusion of a further step, component or feature or group of steps, components or features.

The invention will hereinafter be further described by way of the following non-limiting example and accompanying figures.

Brief description of the accompanying figures:

Figure 1: Shows features of the Na^+-dependent pH_i recovery measured with a zero Na^+ pipette solution. (A) Representative experiment with 10 mM ATP in the pipette. The bar indicates the period of readmission of 155 mM Na^+ solution to the bath. (B) Concentration-response relation for the effect of extracellular ethylisopropylamiloride (EIPA) on the Na^+-dependent pH_i recovery. (C) The effect of modifying intracellular ATP levels.

Figure 2: Shows inhibition of Na^+ -dependent pH_i recovery by cytosolic Na^+. (A) Dependency of the Na^+-dependent pH_i recovery on pipette Na^+. (B) The effects of inclusion of 20 mM $NMDG^+$ in the zero Na^+ pipette solution, or by buffering intracellular and extracellular Ca^{2+} to zero by the inclusion of 20 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetate (BAPTA) in the pipette solution and 1 mM EGTA in the bath solution. No Ca^{2+} was added to either solution.

Figure 3: Shows that the Na^+ feedback inhibition is mediated by a G protein. (A) The effect of the addition of 100 μM GDP-β-S to the pipette solution. (B) The effect of the addition of 500 ng/ml activated pertussis toxin to the pipette solution. (C) The effect of the addition to the pipette solution of antibodies directed against various G protein α-subunits [$AbG_{i1, i2}$ = against

C terminals of $G\alpha_{i1}$ and $G\alpha_{i2}$; $AbG_{o,i3}$ = against C terminals of $G\alpha_o$ and $G\alpha_{i3}$; AbG_{i3} = against C terminal of $G\alpha_{i3}$; AbG_o = against N terminal of $G\alpha_o$; all 1 in 200 (vol/vol)].

Figure 4: Shows the inhibition of Na^+ feedback by intracellular amiloride. (A) Concentration-dependency of the effect of intracellular amiloride when included in 20 mM Na^+ solution. (B) The effect of the inclusion of 0.2 μM activated recombinant α-subunit of G_o (act G_o) and amiloride (10 and 30 μM) in the zero Na^+ pipette solution. AS and inact G_o denote controls in which activation solution or inactive $G\alpha_o$, respectively, were added to the pipette solution. (C) The effect of the inclusion of anti-Nedd4 antibody (A-Nd4; 1 μg purified 1gG/ml). GST-WW fusion protein (G-W; 0.3 mg/ml). GST-wild type-ubiquitin (wt; 0.3 mg/ml) or GST-dominant negative-ubiquitin (K48R) fusion protein (dn; 0.3 mg/ml) in the 20 mM Na^+ pipette solution. In A and C the broken lines indicate the mean rate of pH_i recovery observed with zero Na^+ pipette solution.

Figure 5: Shows the mechanisms of feedback inhibition by intracellular Na^+ of epithelial Na^+ channels in salivary duct (absorptive) cells (A) and Na^+-H^+ exchange in salivary endpiece (secretory) cells (B). In each cell model, the apical membrane is on the left and the sodium pump (Na^+, K^+ ATPase) is shown in the basolateral membrane on the right.

Example 1: Control of Na^+-H^+ exchange in salivary secretory cells by an intracellular Na^+ receptor.

Materials and Methods.

Cell Preparation. Male Quackenbush strain mice were killed by cervical dislocation, and the mandibular glands were removed, finely minced, and incubated for 12 min in a physiological salt solution containing 1 mg/ml collagenase (Worthington type IV). The cell suspension was then dispersed by trituration and washed with fresh Na^+ rich bath solution containing 145 mM NaCl, 5.5 mM KCl, 1.2 mM $MgCl_2$, 7.5 mM Na-Hepes, 7.5 mM H-Hepes, 1 mM $CaCl_2$ and 10 mM glucose; the pH was adjusted to 7.4 with NaOH. The cells were filtered through a 75-μm nylon mesh and kept on ice until required.

Patch-Clamp Techniques. A technique based on that of Demaurex and coworkers (21) was used in which the whole-cell patch-clamp technique is used to control cytosolic composition while the pH-sensitive dye, BCECF, is

used to measure intracellular pH (pH_i). The patch-clamp techniques used were are described (22), and the cells were loaded with BCECF by including it in the pipette solution. Except for the experiments summarised in Figure 1C, in which $MgSO_4$ replaced MgATP, pipettes were filled with solutions containing 145 mM K-glutamate and Na-glutamate combined, 5 mM KCl, 5 mM Mes, 10 mM Mg-ATP, 1 mM EGTA, 40 mM sucrose, and 0.2 mM BCECF; the pH was adjusted to 6.0.

Measurement of pH_i. The equipment used to measure pH_i was as described (23). The chamber (0.3 ml) was continuously perfused with a Na^+-free bath solution containing 145 mM *N*-methyl-D-glucamine (NMDG)-Cl, 5.5 mM KCl, 15 mM H-Hepes, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM glucose with a pH of 7.4. Single cells in the whole-cell configuration were voltage-clamped at -30 mV. After 3 min they were illuminated alternately at 490 and 430 nm. Na^+-H^+ exchange activity was measured by reintroducing Na^+ to the bath between 2 and 3 min after the start of illumination. pH_i recovery rate was determined by fitting a linear regression to the linear phase of the pH_i recovery (i.e., between 20% and 80% of maximal recovery). Calibration of the BCECF signal was by the nigericin high-K^+ method (23).

Chemicals. Sources of chemicals and the methods for activating pertussis toxin and G protein α-subunits were as reported (24, 25). Antibodies directed against the C terminals of the α-subunits of G_{i1}/G_{i2}, G_{i3} and G_{i3}/G_o were obtained from Calbiochem, and antibodies against the N-terminal of the α-subunit of G_o were obtained from DuPont-NEN. They were used in the pipette solution at a 1 in 200 (vol/vol) dilution of the solution provided by the manufacturer. Glutathione-S-transferase (GST)-WW (G-W), GST-dominant negative-ubiquitin (K48R), and GST-wild type-ubiquitin fusion proteins were produced as described (24). The anti-Nedd4 antibody (A-Nd4) was purified IgG raised in rabbits against the C-terminal half of the protein (24, 26).

Results are presented as means ± SEM. At least five cells were tested in each experimental group. Statistical significance was assessed by using Student's unpaired t test. All experiments were performed at 22°C.

Results.

Activity of Na^+-H^+ exchangers was measured by a technique described by Demaurex and coworkers (21) in which the whole-cell configuration of the patch-clamp technique is used to control cytosolic composition while the pH-

sensitive dye, BCECF, measures pH_i. The cells were bathed initially in a zero Na^+ solution so that they would be unable to oppose the acid load imposed by the pipette solution using Na^+-dependent H^+ transporters such as the Na^+-H^+ exchanger. The bath solution then was changed to one containing 155 mM Na^+ so as to activate the Na^+-H^+ exchanger and cause pH_i to recover toward normal levels (Fig 1A). The rate of this Na^+-dependent pH_i recovery was used to estimate Na^+-H^+ exchange activity. The technique was validated by demonstrating that Na^+-dependent pH_i recovery has features consistent with its being the result of the NHE1 isoform of Na^+-H^+ exchanger, which predominates in salivary secretory cells. It was found that the Na^+-dependent pH_i recovery was highly sensitive to the amiloride analog, ethylisopropylamiloride (Fig. 1B), and that the recovery depended on the presence of ATP (21), being inactivated when intracellular ATP was depleted by treatment with 2-deoxy-D-glucose (5 mM) and oligomycin (5 μg/ml; Fig. 1C).

It was demonstrated that the rate of the Na^+-dependent pH_i recovery declined with increasing pipette Na^+ concentration (Fig. 2A) in a manner similar to that described in sheep F2 Purkinje fibres (27). This inhibition evidently was caused by increased $[Na^+]_i$, because it could not be reproduced by the large organic cation, $NMDG^+$ (Fig. 2B). Because intracellular free Ca^{2+} is known to regulate Na^+-H^+ exchangers (28), an investigation was made to determine whether a change in free intracellular Ca^{2+} concentration could mediate this phenomenon. It was found that buffering cytosolic and extracellular Ca^{2+} to nominal zero did not alter the effect of increased $[Na^+]_i$ (Fig. 2B).

An investigation was also made to determine the mechanism by which $[Na^+]_i$ controls the activity of the Na^+-H^+ exchanger. It was found that inclusion of the pipette solution of 100 μM GDP-β-S (which competitively inhibits the binding of GTP by G proteins; ref. (29) or of 500 ng/ml activated pertussis toxin (which ADP ribosylates G proteins of the G_i and G_o classes so as to prevent their interaction with receptors; ref. (30), reversed the inhibitory effect of 20 mM Na^+ (Fig. 3A and B). The ability of these agents to overcome the inhibitory effect of raised intracellular Na^+ completely without altering the electrochemical gradient for Na^+ indicates that the inhibition is not caused by a decreased electrochemical driving force for Na^+-H^+ exchange. Rather, it must be caused by a G protein-mediated feedback

pathway. In this regard, it was further found that inclusion in the pipette solution of antibodies directed against the α-subunit of the G_o protein, which is known to be expressed in salivary endpiece cells (31), abolished the inhibitory effect of 20 mM Na^+. In contrast, antibodies directed against the α-subunits of G_{i1}, G_{i2}, and G_{i3} were without effect (Fig. 3C).

In the absorptive cell of the salivary duct, $[Na^+]_i$ is sensed by a receptor the effect of which is mediated by G_o (10). This receptor is blocked by amiloride and amiloride analogs such as dimethylamiloride and benzimidazolylguanidinium, thus explaining the ability of these agents to stimulate Na^+ channel activity. It was found that the inclusion of amiloride in the pipette solution reversed the inhibitory effect of 20 mM Na^+ (Fig. 4A). Further, it was found that the inclusion of the activated α-subunit of G_o in the zero Na^+ pipette solution (Fig. 4B) inhibited the Na^+-H^+ exchanger and that the inclusion of as much as 30 μM amiloride in the pipette solution was unable to overcome this inhibition (Fig. 4B). Thus, amiloride exerts its inhibitory action upstream of G_o, presumably at the putative receptor for intracellular Na^+.

Discussion.

It has been previously shown that $[Na^+]_i$ and the G protein, G_o, regulate the activity of the epithelial Na^+ channel in the duct cells of the mouse mandibular gland via Nedd4 (24), a ubiquitin-protein ligase that is believed to bind to Na^+ channels and regulate their activity by ubiquitinating them (12, 13). Here, it was found that feedback inhibition of the Na^+-H^+ exchanger was not prevented by inclusion in the pipette solution of an antibody directed against Nedd4 or of a fusion protein composed of GST and the three WW-domains of mouse Nedd4 (GST-W), which acts as a dominant negative mutant of Nedd4 (Fig. 4C). This finding is consistent with the low level of expression of Nedd4 in endpiece cells (24). Feedback inhibition was blocked, however, by inclusion of a dominant negative mutant of ubiquitin (K48R) (24) in the pipette solution (Fig. 4C), indicating that feedback regulation of the exchanger nevertheless is mediated by ubiquitination. Because our preliminary data show that NHE1 transfected into COS cells is ubiquitinated (data not shown), the findings indicate that feedback regulation of NHE1 is mediated by ubiquitination of the exchanger protein. The control system then would resemble the control of surface expression of epithelial Na^+ channels by ubiquitination of the channel protein catalysed by Nedd4.

It cannot be excluded however, that the inactivation of NHE1 produced by Na^+ feedback is the result of ubiquitination of a protein associated with the exchanger, as recently has been proposed for the control of the growth hormone receptor by ubiquitination (32). Whatever the mechanism, the present findings taken together with the finding that activity of epithelial Na^+ channels can be rapidly down-regulated by ubiquitination suggest that ubiquitination may be a general mechanism for the rapid control of membrane transport protein activity.

Example 2: Prevention of the progression of diabetic nephropathy and other forms of chronic renal failure by 6-iodoamiloride.

Materials and methods.

20 mg 6-iodoamiloride tablets may be formulated and taken orally at a dosage of one or two every 6 hours.

Discussion.

6-iodoamiloride acts by blocking the intracellular Na^+ receptor that controls NHE1 and other sodium-dependent transporters as well as mediating the normal cellular responses to increased intracellular sodium concentration (which include release of cytokines and increased cell growth and proliferation (44). In this way, cytokine release and cellular proliferation caused by increased intracellular sodium can be treated with 6-iodoamiloride to prevent the cytokine release and cell growth and proliferation that lead to progression of renal failure.

Example 3: Treatment of cells with reduced Na^+ transport with recombinant adenovirus.

Materials and methods.

Recombinant adenovirus. Recombinant adenovirus including an expressible gene encoding the Na^+ receptor, GILT may be prepared by routine molecular biology techniques (33). Particularly, the clone encoding GILT (SEQ ID NO:1) may be ligated to a suitable mammalian promoter sequence (e.g. CMV (45)) and inserted into a suitable vector for the transfer, by homologous recombination, of the recombinant GILT gene into an adenovirus as described by He et al. (46).

Administration. The recombinant adenovirus may be formulated and administered in accordance with known methods in the art. In particular,

the recombinant adenovirus may be formulated for administration as a nasal spray or intrabronchial spray or given intraveneously (47, 48) or direct injections of muscle or of organs (49). With administration to the respiratory tract (50), the recombinant adenovirus will preferably be administered at a dose of 10^9 plaque forming units (pfu) at intervals between 2 and 4 weeks.

Discussion.

Upon infection of host diseased cells, the adenovirus will bring about the expression of functional GILT protein to decrease Na^+ transport and restore cytosolic ion composition to substantially that of corresponding healthy cells.

Example 4: Prevention of the progression of chronic hypoxic pulmonary hypertension and other forms of pulmonary hypertension by 6-iodoamiloride and other inhibitors of the sodium receptor.

Materials and methods.

20mg 6-iodoamiloride tablets may be formulated and taken orally at a dosage of 1 or 2 every 6 hours.

Discussion.

6-iodoamiloride acts by blocking the intracellular Na^+ receptor that controls NHE1 and other sodium-dependent transporters as well as mediating the normal cellular responses to increased intracellular sodium concentration (which include release of cytokines and increased cell growth and proliferation (44). In this way, cytokine release and cellular proliferation caused by increased intracellular sodium can be treated with 6-iodoamiloride to prevent the cytokine release and cell growth and proliferation that lead to progression of pulmonary hypertension due to chronic hypoxia (51).

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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Claims:

1. A method of treatment of a human disease which is characterised by abnormal cytosolic ion composition in diseased cells resulting from reduced or over activity of a Na^+ transport protein, the method comprising administering to a subject having said disease an effective amount of an agent that substantially restores the ion composition of the cytosol in said diseased cells to that which is found in corresponding cells from healthy tissue.

2. A method according to claim 1, wherein the Na^+ transport protein is other than an epithelial Na^+ receptor.

3. A method according to claim 1, wherein the Na^+ transport protein is selected from the group consisting of Na^+-H^+ exchanger 1 (NHE1), Na^+-H^+ exchanger 2 (NHE2), Na^+-H^+ exchanger 3 (NHE3), the Na^+-HCO_3^- cotransporter and the Na^+K^+2Cl cotransporter.

4. A method according to any one of claims 1-3, wherein the abnormal cytosolic ion composition in diseased cells arises from reduced Na^+ transport protein activity.

5. A method according to claim 4, wherein said agent is selected from gene therapy agents and blocking agents of the Na^+ transport protein inhibitory feedback mechanism.

6. A method according to claim 5, wherein said agent is a recombinant adenovirus including a nucleotide sequence encoding a non-mutated Na^+ transport protein which is other than a non-mutated epithelial Na^+ receptor.

7. A method according to claim 5, wherein the agent is selected from amiloride and amiloride analogs.

8. A method according to claim 5, wherein said agent is a G-protein inhibitor.

9. A method according to claim 8, wherein the G-protein inhibitor is selected from GDP-β-S and NF023.

10. A method according to claim 5, wherein said agent is a ubiquitin protein ligase inhibitor.

11. A method according to claim 10, wherein the ubiquitin protein ligase inhibitor is selected from dominant negative mutants of ubiquitin and agents that prevent binding of ubiquitin protein ligase to Na^+ transport proteins.

12. A method according to any one of claims 1-3, wherein the abnormal cytosolic ion composition in diseased cells arises from over activity of a Na^+ transport protein.

13. A method according to claim 12, wherein said agent is selected from gene therapy agents, intracellular Na^+ receptor activators; G-protein activators, ubiquitin ligase activators and endocytosis triggering agents.

14. A method according to claim 13, wherein said gene therapy agent is an adenovirus including a nucleotide sequence encoding a non-mutated protein which participates in the Na^+ transport protein inhibitory feedback mechanism.

15. A method according to claim 13, wherein said intracellular Na^+ receptor activator is selected from guanidium and guanidium analogs.

16. A method according to claim 13, wherein said G-protein activator is selected from GDP-γ-S and receptor mimetic peptides.

17. A method of diagnosis of a human disease which is characterised by abnormal cytosolic ion composition in diseased cells resulting from reduced or over activity of a Na^+ transport protein, the method comprising isolating from a subject suspected of having said disease a sample of cells and assessing said sample of cells for reduced or over activity of said Na^+ transport protein or the Na^+ transport protein in inhibitory feedback mechanisms.

18. A method according to claim 17, wherein said Na^+ transport protein is other than an epithelial Na^+ receptor.

19. A method according to claim 17, wherein said Na^+ transport protein is selected from the group consisting of Na^+-H^+ exchanger 1 (NHE1), Na^+-H^+ exchanger 2 (NHE2), Na^+-H^+ exchanger 3 (NHE3), the Na^+-HCO_3^- cotransporter and the Na^+K^+2Cl cotransporter.

20. A method according to any one of claims 17 to 19, wherein the sample of cells is a sample of epithelial cells or lymphocytes.

21. A method according to any one of the claims 17 to 20, wherein the sample of cells is assessed for reduced or over activity of Na^+ transport protein by determining the rate of Na^+-dependent intracellular pH (pH_i) recovery and comparing said rate against similarly measured rates from cells from healthy tissue isolated from said subject or a control subject(s).

22. A method of diagnosis of a human disease which is characterised by abnormal cytosolic ion composition in diseased, the method comprising isolating from a subject suspected of having said disease a sample of cells and assessing said sample of cells for over or under expression of the Na^+ transport protein or another protein participating in the Na^+ transport protein inhibitory feedback mechanism.

23. A method of diagnosis of a human disease which is characterised by abnormal cytosolic ion composition in diseased cells resulting from reduced or over activity of a Na^+ transport protein, the method comprising isolating a genomic DNA sample from a subject suspected of having said disease and assessing said sample for the presence of a gene encoding a mutated product causative of said reduced or over activity of said Na^+ transport protein.

24. A method of assessing a subject for a predisposition to a human disease which is characterised by abnormal cytosolic ion composition in diseased cells resulting from reduced or over activity of Na^+ transport protein, the method comprising isolating a genomic DNA sample from a

subject and assessing said sample for the presence of a gene encoding a mutated product causative of said reduced or over activity of said Na^+ transport protein.

25. A method according to any one of claims 22 or 24, wherein the said Na^+ transport protein is other than an epithelial Na^+ receptor.

26. A method according to any one of claims 22 to 24, wherein said Na^+ transport protein is selected from the group consisting of Na^+-H^+ exchanger 1 (NHE1), Na^+-H^+ exchanger 2 (NHE2), Na^+-H^+ exchanger 3 (NHE3), the Na^+-HCO_3^- cotransporter and the Na^+K^+2Cl cotransporter.

27. A method according to any one of claims 1 to 26, wherein the said human disease is selected from hypertension, renal failure, cardiac hypertrophy and cardiological syndrome X.

28. An isolated DNA molecule encoding an intracellular Na^+ receptor designated GILT, said DNA molecule comprising a nucleotide sequence showing ≥ 75% homology to the nucleotide sequence shown as SEQ ID NO: 1.

29. A DNA molecule according to claim 28, wherein said DNA molecule comprises a nucleotide sequence showing ≥ 85% homology to the nucleotide sequence shown as SEQ ID NO: 1.

30. A DNA molecule according to claim 28, wherein said DNA molecule comprises a nucleotide sequence showing ≥ 95% homology to the nucleotide sequence shown as SEQ ID NO: 1.

31. A DNA molecule according to claim 28, wherein said DNA molecule comprises a nucleotide sequence substantially corresponding to that shown as SEQ ID NO: 1.

32. A DNA molecule according to claim 28, wherein said DNA molecule encodes a protein comprising an amino acid sequence substantially corresponding to that shown as SEQ ID NO: 2.

33. An isolated DNA molecule encoding an intracellular Na^+ receptor designated SCunique, said DNA molecule comprising a nucleotide sequence showing ≥ 75% homology to the nucleotide sequence shown as SEQ ID NO: 3.

34. A DNA molecule according to claim 33, wherein said DNA molecule comprises a nucleotide sequence showing ≥ 85% homology to the nucleotide sequence shown as SEQ ID NO: 3.

35. A DNA molecule according to claim 33, wherein said DNA molecule comprises a nucleotide sequence showing ≥ 95% homology to the nucleotide sequence shown as SEQ ID NO: 3.

36. A DNA molecule according to claim 33, wherein said DNA molecule comprises a nucleotide sequence substantially corresponding to that shown as SEQ ID NO: 3.

37. A DNA molecule according to claim 33, wherein said DNA molecule encodes a protein comprising an amino acid sequence substantially corresponding to that shown as SEQ ID NO: 4.

38. A host cell transformed with a DNA molecule according to any one of claims 28 to 37.

39. A method of producing an intracellular Na^+ receptor, comprising culturing the host cell of claim 38 under conditions enabling the expression of said DNA molecule and optionally recovering the expressed receptors.

40. An intracellular Na^+ receptor designated GILT, said receptor comprising an amino acid sequence substantially corresponding to that shown as SEQ ID NO: 2, in a substantially pure form.

41. An intracellular Na^+ receptor designated SCunique, said receptor comprising an amino acid sequence substantially corresponding to that shown as SEQ ID NO: 4, in a substantially pure form.

42. An antibody which specifically binds to a receptor according to claim 40 or 41.

43. A method for detecting agonist or antagonist agents of the receptor of claim 40 or 41, wherein said method comprises contacting said receptor, or a host cell transformed with and expressing the DNA molecule of any one of claims 28 to 37, with a test agent under conditions enabling the activation of said receptor, and detecting an increase or decrease in activity of the receptor.

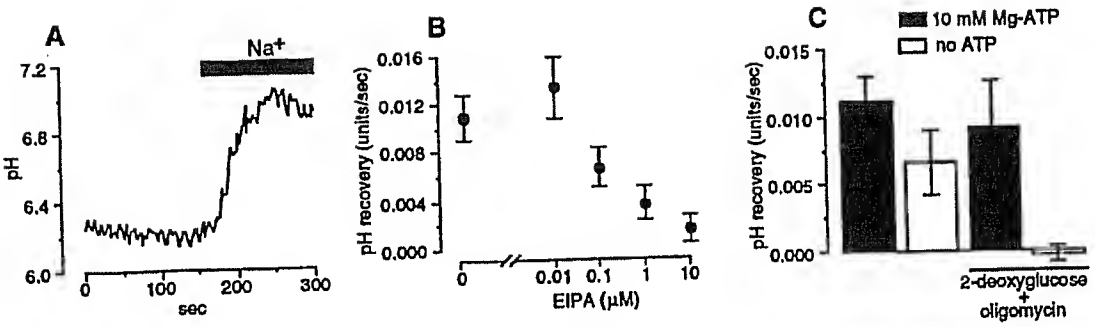
44. A nucleic acid probe or primer comprising a nucleotide sequence of 10 or more nucleotides, wherein said probe or primer specifically hybridises to a unique sequence within the DNA molecule of claim 31 or 36 under high stringency conditions.

45. An isolated DNA molecule comprising a nucleotide sequence substantially corresponding to that shown as SEQ ID NO: 5.

46. An isolated DNA molecule comprising a nucleotide sequence substantially corresponding to that shown as SEQ ID NO: 6.

47. An isolated DNA molecule comprising a nucleotide sequence substantially corresponding to that shown as SEQ ID NO: 7.

Figure 1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0.008
0.004
0.000
pH recovery (units/sec)
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0.01
0.1
1
10
EIPA (μM)
C
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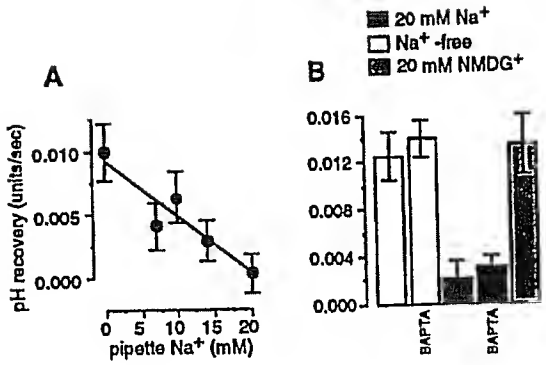
+
oligomycin

Figure 2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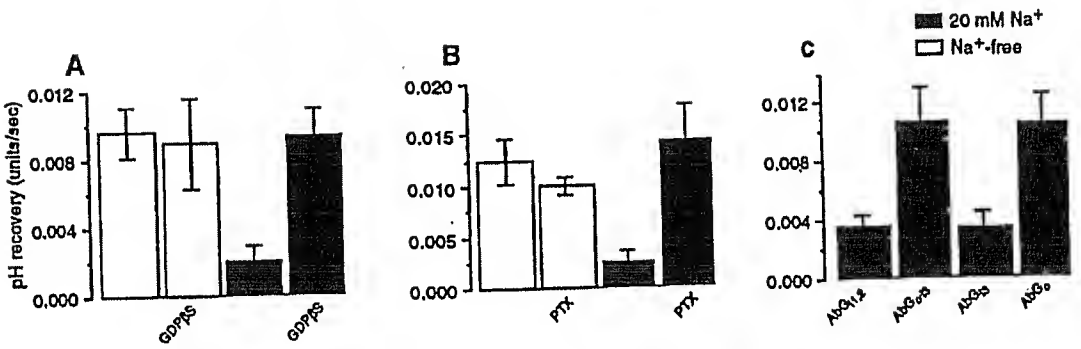
BAPTA

Figure 3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PTX

Figure 4

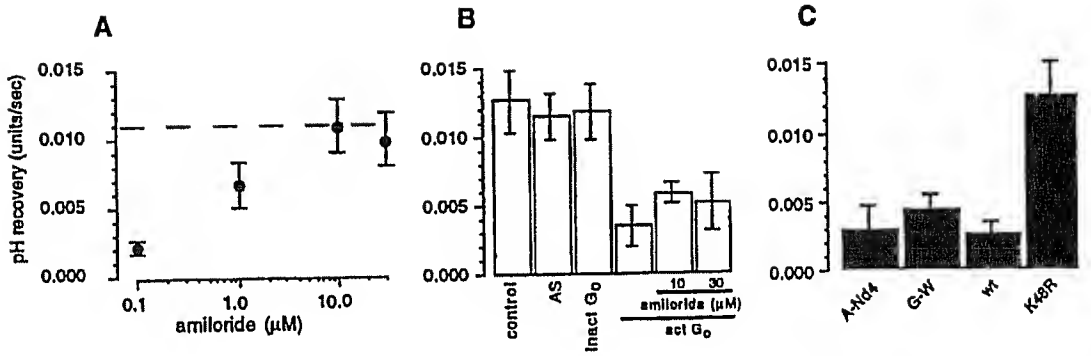
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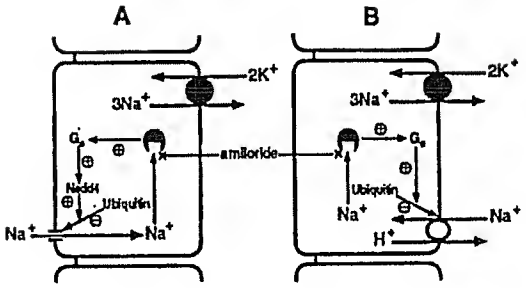
Figure 5



Sequence Listing:

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/00980

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C12N 15/12; A61K 38/16, 48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
SEE ELECTRONIC DATABASE BOX BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
SEE ELECTRONIC DATABASE BOX BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See extra sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	Takesono, A. et al (1999) "Receptor-independent activators of heterotrimeric G-protein signaling pathways" J. Biol. Chem. 274(47), pages 33202-5. See the entire document.	28-32, 40, 45, 46
PX	Ishibashi, H. et al (1999) Na^+-H^+ exchange in salivary secretory cells is controlled by an intracellular Na^+ receptor" Proc. Natl. Acad. Sci. USA 96, pages 9949-53 See the entire document	3, 5-11, 14-16, 19, 26

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 October 2000	23 OCT 2000
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **TERRY MOORE** Telephone No: (02) 6283 2632

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/00980

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Harvey, K.F. et al (1999) "All three WW domains of murine Nedd4 are involved in the regulation of epithelial sodium channels by intracellular Na^+" J. Biol. Chem. 274(18), pages 12525-30 See in particular the discussion.	5, 10, 11, 14
X	Komwatana, P. et al (1998) "Activators of epithelial Na^+ channels inhibit cytosolic feedback control. Evidence for the existence of a G protein-coupled receptor for cytosolic Na^+" J. Membrane Biol. 162, pages 225-32 See in particular the discussion and figure 7.	5, 7, 8, 15, 16
X	Dinudom, A. et al (1998) "Nedd4 mediates control of an epithelial Na^+ channel in salivary duct cells by cytosolic Na^+" Proc. Natl. Acad. Sci. USA 95, pages 7169-73 See in particular the discussion and figure 4.	5, 6, 10, 11, 14
X	Cook. D.I. et al (1998) "Control of Na^+ transport in salivary duct eptithelial cells by cytosolic Cl^- and Na^+" Eur. J. Morphology 36, Supplement ++pages 67-73 See in particular the summary and figure 8.	5, 7-9, 14, 16
X	Symons, J.D. et al (1998) "Na-H exchange inhibition with cariporide limits functional impairment caused by repetitive ischemia" J. Cardiovascular Pharmacology 32, pages 853-62 See the entire document.	3, 5, 7
X	EP A 726 254 (MITSUI TOATSU CHEMICALS, INC.) 14 August 1996 See page 2, line 120 - page 3, line 3 and claim 10.	3, 5, 7
X	Komwatana, P. et al (1996) "Cytosolic Na^+ controls an epithelial Na^+ channel via the G_o guanine nucleotide-binding regulatory protein" Proc. Natl. Acad. Sci. USA 93, pages 8107-11 See in particular the discussion and figure 5.	5, 7, 8, 9, 14, 16
X	EP A 622 356 (SUMITOMO PHARMACEUTICALS CO., LTD.) 2 November 1994 See in particular page 3.	3, 5, 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/00980

Box I **Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos :
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos :
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos :
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II **Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

See extra sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
3, 5-11, 14-16, 19, 26 and 28-47

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/00980

Supplemental Box
(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box No: II

The international application contains 8 separate inventions. These are:

Invention 1. Regulating the activity of NHE1 for normalising cytosolic ion concentration.

Invention 2 Regulating the activity of NHE2 for normalising cytosolic ion concentration.

Invention 3. Regulating the activity of NHE3 for normalising cytosolic ion concentration.

Invention 4. Regulating the activity of Na^+HCO^- cotransporter for normalising cytosolic ion concentration.

Invention 5. Regulation the activity of $Na^+K^+2Cl^-$ cotransporter for normalising cytosolic ion concentration.

Invention 6. Regulating the activity of any Na^+ receptor for normalising cytosolic ion concentration.

Invention 7. Sequences 1, 2, 5 and 6 defining GILT sequences and variants.

Invention 8. Sequences 3, 4 and 7 defining SCunique sequences and non-coding regions thereof.

The feature linking inventions 1-6 resides in the regulation of Na^+ receptors for therapeutic use. However using epithelial Na^+ receptors for therapeutic use is already known. Thus a unity of invention does not exist *a posteriori*.

Inventions 7 and 8 are directed to the identification of new and alternative Na^+ receptors. The only common feature between these two inventions and inventions 1-6 is the Na^+ receptors. Na^+ receptors are already known (see above) and thus no unity exists *a posteriori*. There does not appear to be any common structural feature linking the two sequences defined in inventions 7 and 8 and thus there is no unity of invention *a priori* between these two groups of sequences.

The ISA searched inventions 7 and 8 under one search fee as it does not seem that significant extra effort is involved in combining these two inventions.

An additional search fee was paid and inventions 1-5 were searched under this search fee because it appeared that a single search could be drafted that would encompass all 5 inventions.

As a result the two searches covered the material defined in claims 3, 5-11, 14-16, 19, 26 and 28-47.

Continuation of Box No: B

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Medline, CA, GenPept, SwissProt, EMBL, Genbank: Sequences 1-7, keywords: GILT, SCunique, NHE1, NHE2, NHE3, Na+/H+ exchanger, Na+HCO- cotransporter, NaKCl cotransporter, sodium channel, cytosolic, intracellular, inhibition, feedback, regulation

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU00/00980

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
EP	622 356	JP	7010839	CN	1106800	CA	2121391
EP	726 254	US	5 627 193	JP	8277269		

END OF ANNEX